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NKG2D-based CAR-T cells and radiotherapy exert synergistic efficacy in glioblastoma

Tobias Weiss[1], Michael Weller[1], Matthias Guckenberger[3], Charles L. Sentman[2], Patrick Roth[1]*

[1]Department of Neurology and Brain Tumor Center, University Hospital Zurich and University of Zurich, Switzerland; [2]Center for Synthetic Immunity and Department of Microbiology & Immunology, Geisel School of Medicine, New Hampshire, USA; [3]Department of Radiation Oncology, University Hospital Zurich and University of Zurich, Switzerland

*Corresponding author: Dr. Patrick Roth, Department of Neurology, University Hospital and University of Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland, Tel.: +41 (0)44 255 5511, Fax: +41 (0)44 255 4380, E-mail: patrick.roth@usz.ch

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Keywords: CAR T cell, NKG2D, immunotherapy, glioblastoma, radiotherapy

Abstract

Chimeric antigen receptor (CAR) T cell therapy is an emerging immunotherapy against several malignancies including glioblastoma, the most common and most aggressive malignant primary brain tumor in adults. The challenges in solid tumor immunotherapy comprise heterogenously expressed tumor target antigens and restricted trafficking of CAR T cells to and impaired long-term persistence at the tumor site, as well as the unaddressed integration of CAR T cell therapy into conventional anti-cancer treatments. We addressed these questions using a NKG2D-based chimeric antigen receptor construct (chNKG2D) in fully immunocompetent orthotopic glioblastoma mouse models. ChNKG2D T cells demonstrated high IFN-γ production and cytolytic activity *in vitro*. Upon systemic administration *in vivo*, chNKG2D T cells migrated to the tumor site in the brain, did not induce adverse events, prolonged survival, and cured a fraction of glioma-bearing mice. Surviving mice were protected long-term against tumor re-challenge. Mechanistically, this was not solely the result of a classical immune memory response, but rather involved local persistence of chNKG2D T cells. A subtherapeutic dose of local radiotherapy in combination with chNKG2D T cell treatment resulted in synergistic activity in 2 independent syngeneic mouse glioma models by promoting migration of CAR T cells to the tumor site and increased effector functions. We thus provide preclinical proof-of-concept of NKG2D CAR T cell activity in mouse glioma models and demonstrate efficacy, long-term persistence, and synergistic activity in combination with radiotherapy, providing a rationale to translate this immunotherapeutic strategy to human glioma patients.

Introduction

Glioblastoma is the most common maligant primary brain tumor in adults (1). It remains one of the most challenging cancers and has still a poor prognosis despite multimodal treatment regimens comprising surgery, radiotherapy and chemotherapy with temozolomide (2,3). Therefore, novel treatment modalities are urgently needed. Adoptive immunotherapy with genetically engineered T cells that express a chimeric antigen receptor (CAR) is an emerging treatment strategy that may also hold promise for neoplasms in the central nervous system (CNS) (4).

The design of chimeric antigen receptors, which consist of an extracellular tumor antigen-binding domain linked to hinge, transmembrane and intracellular signaling domains (5,6), allows customized T cell engineering and an efficient anti-tumor response of bulk T cells in a major histocompatibility complex (MHC)-independent manner (7). CAR T cell therapy has led to encouraging clinical responses in hematological malignancies (8,9) and is also studied in solid tumors including glioblastoma (10). However, in solid tumors there are several challenges that hamper the efficacy of CAR T cell therapy which need to be addressed, such as the identification of homogeneously expressed tumor-associated or tumor-specific target antigens, the migration of CAR T cells to the tumor site, and the immunosuppressive microenvironment that may impede the function and persistence of CAR T cells (11). CAR T cell strategies that are currently explored against glioblastoma target single tumor antigens such as epidermal growth factor receptor variant III (EGFRvIII) (12), erythropoietin-producing hepatocellular carcinoma A2 (EphA2) (13), human epidermal growth factor receptor 2 (Her2) (14,15) or interleukin 13 receptor subunit alpha 2 (IL13Rα2) (16). These targets are non-homogeneously expressed and susceptible to antigen escape (17).

We and others have assessed the importance of the natural-killer group 2-member D (NKG2D) system in glioblastoma (18-22), which has unique features such as the promiscuous binding properties of the NKG2D receptor to multiple tumor-associated NKG2D ligands and the inducibility of these ligands on the tumor cell surface by chemotherapy and radiotherapy (23). NKG2D-based CAR T cells elegantly use the favorable properties of the NKG2D system. The NKG2D CAR design comprises the full length NKG2D protein fused to CD3ζ and it associates with DNAX-activation protein 10 (DAP10) at the cell surface. This NKG2D-CD3ζ-DAP10 complex functionally acts as a second-generation CAR which provides a T cell activation signal through CD3ζ and co-stimulation through DAP10 (24,25). NKG2D CAR T cells have never been tested against intracranially growing tumors such as gliomas. Furthermore, the combination of CAR T cell therapy with conventional treatment regimens has never been examined, but the inducibility of NKG2D ligands by various stressors provides a strong rationale for this approach.

Here, we investigated NKG2D-based CAR T cells in orthotopic, syngeneic glioma models and addressed the questions of efficacy, trafficking, persistence and combination with conventional anticancer therapy in fully immuno-competent hosts.

Material and Methods

Cell lines

SMA glioma cell lines were obtained from Dr. D. Bigner (Duke University Medical Center, Durham, North Carolina, USA) and GL-261 cells were obtained from the National Cancer Institute (Frederick, Maryland, USA). EL-4 cells were obtained from from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Basel, Switzerland), containing 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), and 10% fetal calf serum (FCS, Biochrom KG, Zug, Switzerland) and regularly tested negative for mycoplasma by PCR. Cells were authenticated routinely at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany by short tandem repeat analysis, lastly in 2013.

CAR design and generation of CAR T cells

The design of the murine NKG2D-based CAR (chNKG2D) and the corresponding control construct that overexpresses wildtype-NKG2D without the intracellular CD3ζ domain (wtNKG2D) has been described (25). CAR T cells were generated by retroviral transduction (25). In short, syngeneic splenocytes from C57BL/6 or VM/Dk mice were activated with 1 µg/ml concanavalin A (Sigma-Aldrich, Steinheim, Germany) for 18-20 h and retrovirally transduced with chNKG2D or wtNKG2D. The cells were maintained in RPMI1640 (Gibco Life Technologies, Paisley, UK) supplemented with 10% FCS, 10 mmol/l HEPES, 2 mM L-glutamine, 1 mmol/l pyruvate, 0.1 mmol/l non-essential amino acids (all from Gibco), 50 µmol/l 2-mercaptoethanol (Sigma-Aldrich) and 25 IU/ml recombinant murine IL-2 (PeproTech, London, UK) for 6-8 days and subsequently used for experiments.

Antibodies and flow cytometry

The following monoclonal antibodies were used for flow cytometry: anti-CD4-AF700, anti-CD8-APC, anti-IFN-γ-BV421, anti-CD45.1-AF488 and anti-CD45.2-APC (BioLegend, San Diego, California, USA). For blocking of FC receptors, samples were preincubated with anti-mouse CD16/CD32 (Biolegend). As controls, we used isotype-matched antibodies from Sigma-Aldrich. Acquisition was performed on a BD FACSVerse Analyzer (BD, Allschwil, Switzerland) and data were analyzed with FlowJo (Tree Star, Stanford, California, USA).

Cytotoxicity assay

Glioma cells as target cells were co-cultured for 4 h with chNKG2D or wtNKG2D T cells at different effector : target ratios. Glioma cell lysis was assessed by a flow cytometry-based assay (18). Specific lysis was expressed as percentage of death of labeled target cells. For blocking experiments, transduced T cells were preincubated for 2 h at 4 °C with anti-NKG2D or isotype control from eBioscience (San Diego, California, USA).

Immunohistochemistry

Staining of brain cryosections from tumor-bearing mice has been described in detail (26). Anti-CD45.1 and anti-CD45.2 antibodies for immunohistochemistry were obtained from Novus Biologicals (Littleton, Colorado, USA). The Histofine Simple Stain Mouse MAX PO was obtained from Nichirei (Tokyo, Japan) and used as secondary antibody system.

Real-time PCR

RNA isolation and cDNA preparation were performed as described (27). Gene expression was measured in an QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, California, USA) with SYBR Green from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The following primers (Microsynth AG, Balgach Switzerland) were used: chNKG2D (28): forward 5′-GGCGTCGACACCATGAGAGCAAAATTCAGCAGGAG-3′, reverse 5′-GGCGCTCGAGTTACACCGCCCTTTTCATGCAGAT-3′, mouse HPRT1: forward 5′-TTGCTGACCTGCTGGATTAC-3′, reverse 5′- TTTATGTCCCCCGTTGACTG-3′ respectively. The conditions were 40 cycles at 95 °C/15 s and 60 °C/1 min. Relative quantification of gene expression was calculated with the ddCT method (29) for relative quantification compared to the housekeeping gene HPRT1.

IFN-γ ELISPOT assay

Individual spleen samples (N=3) were obtained from NKG2D CAR T cell-treated long-term survivors 8 months after initial treatment or naive control mice. Untouched T cells were isolated using Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and 2 x 10^6 T cells were co-cultured with GL-261 cells for 24 h. The number of IFN-γ secreting cells was measured using mouse IFN-γ ELISPOT Ready-SET-Go from eBioscience and spot forming cells (SFC) were counted using AID EliSpot Reader classic (AID GmbH, Strassberg, Germany).

IFN-γ ELISA

NKG2D CAR T cell-treated long-term survivors, 12 months after initial treatment, or naïve control mice were (re-)challenged with intracranial implantation of GL-261 cells. Three days after tumor implantation, mice were euthanized, splenocytes were isolated and brain-resident $CD4^+$ or $CD8^+$ T cells were FACS-sorted. Subsequently, 5 x 10^4 splenocytes from long-term surviving mice or naïve control mice were co-cultured with 2.5 x10^4 GL-261 or EL-4 cells and 5 x 10^3 FACS-sorted brain-resident $CD4^+$ or $CD8^+$ T cells were co-cultured with 2.5 x 10^3 GL-261 or EL-4 cells. After 72 h, cell-free conditioned media was assessed for IFN-γ by ELISA from Thermo Fisher Scientific.

Animal experiments

All experiments were done in accordance with the Institutional Animal Care and Use Committee (IACUC) of the cantonal veterinary office (ZH006/15) and according to guidelines of the Swiss federal law on animal protection. Wild-type C57BL/6 CD45.2 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and C57BL/6 CD45.1 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). VM/Dk mice were bred in pathogen-free facilities at the University of Zurich. Mice of 6 to 12 weeks of age were used in all experiments. For intracranial tumor implantation, GL-261 cells (2 x 10^4) were stereotactically implanted into the right striatum at day 0. Mice were observed daily and sacrificed as indicated or when developing neurological symptoms. Where indicated, mice received 5 x 10^6 chNKG2D or wtNKG2D T cells intravenously at days 5, 7 and 10 after tumor implantation. For local administration, up to 2 x 10^6 transduced T cells were injected intratumorally at day 5 after tumor implantation. If indicated, local cranial radiotherapy with a single dose of 4 Gy was applied at day 7 after tumor implantation using a Gulmay 200 kV X-ray unit at 1 Gy/min. Long-term surviving mice were re-challenged

5-8 months after the initial tumor inoculation with another tumor implantation in the contralateral hemisphere without any further treatment. For isolation of tumor-infiltrating immune cells at indicated time points, we perfused mice with ice-cold PBS to remove all circulating leukocytes from the CNS and isolated the brains. Subsequently, tumor cells were separated from myelin and red blood cells using a Percoll gradient suspension (Sigma-Aldrich). Cells were washed with PBS and stained with Zombie Aqua™ Fixable Viability Kit (Biolegend) and fluoro-conjugated antibodies specific to cell surface markers for flow cytometry as indicated.

Magnetic resonance imaging (MRI)

MRI was performed with a 4.7 T imager (Bruker Biospin, Ettlingen, Germany) at day 15 after tumor implantation. Coronal T2-weighted images were acquired using Paravision 6.0 (Bruker BioSpin). If indicated, mean and SD of the tumor volume in mm^3 from 5 mice/group were calculated using the formula (length x width x depth)/2.

Fluorescence molecular tomography (FMT)

Transduced T cells were labeled with CellBriteTM NIR790 (Biotium, Fremont, California, USA) and administered to tumor-bearing mice as indicated. For FMT imaging at indicated time points after T cell injection, mice were anesthetized by gas anesthesia, depilated at the tumor region, placed in a FMT4000 system (PerkinElmer, Waltham, Massachusetts USA) and scanned with the 790 nm laser channel. Image analysis was performed using TrueQuant 3.1 (PerkinElmer). A region of interest of equal size was placed above the tumor region and fluorescence intensity was automatically calculated by the software.

Statistical analysis

Data are presented as means and SD. Experiments were repeated at least three times, if not indicated differently. Statistical analyses were performed in GraphPad Prism (La Jolla, California, USA) using multiple two-tailed Student's t-tests and correction for multiple comparisons using the Holm-Sidak method. Kaplan Meier survival analysis was performed to assess survival differences among the treatment groups and p values were calculated with the log-rank test. Significance was concluded at $*p < 0.05$ and $**p < 0.01$.

Results

ChNKG2D-transduced T cells lyse glioma cells in a NKG2D-dependent manner

We generated murine chNKG2D CAR T cells or wtNKG2D overexpressing T cells by retroviral transduction of splenocytes derived from C57BL/6 or VM/Dk mice. NKG2D cell surface levels in splenocytes transduced with chNKG2D or wtNKG2D were equivalent (Suppl. Figure 1). The differential biological effects exerted by these cells can therefore be attributed specifically to the chimeric construct. To determine the cytolytic activity of chNKG2D- or wtNKG2D-transduced T cells, we used these cells as effector cells and different syngeneic murine glioma cell lines as target cells in cytotoxicity assays. ChNKG2D T cells had a significantly higher specific cytolytic activity against all murine glioma cell lines than wtNKG2D T cells (Figure 1A-D, upper panels). Inhibition of NKG2D signaling using a blocking antibody abrogated the enhanced cytolysis, confirming the NKG2D dependency of chNKG2D T cells (Fig. 1A-D, lower panels). Because cytokines are important effector molecules of CAR T cell function, we assessed the T cell specific IFN-γ production of chNKG2D or wtNKG2D T cells co-cultured with syngeneic glioma cells by intracellular cytokine staining. Both $CD4^+$ and $CD8^+$ T cells produced more IFN-γ after transduction with chNKG2D compared to wtNKG2D when the T cells were co-cultured with syngeneic glioma cells (Figure 2A-D).

NKG2D-based CAR T cells home to orthotopic gliomas after systemic administration

The concept of CNS immune privilege has been refined by the demonstration of an intact afferent arm of CNS-related immunity which includes the discovery of classical lymphatic vessels in the meninges (30). However, the efferent arm is restricted, i.e. leukocyte migration to the brain (31) and the immunosuppressive microenvironment of solid tumors such as gliomas is another challenge for access of CAR T cells to the tumor site. To determine whether NKG2D-based CAR T cells reach orthotopically growing gliomas after systemic administration, we labeled chNKG2D T cells with a near-infrared dye that allows *in vivo* tracking by fluorescence molecular tomography (FMT). After a single intravenous injection of 5 x 10^6 chNKG2D T cells, we detected a FMT signal at the orthotopic tumor site that increased over several days (Figure 3A). Labeled wtNKG2D T cells also reached the tumor site (Suppl. Figure 2). In order to corroborate this finding, we injected chNKG2D-transduced T cells generated from splenocytes of CD45.1$^+$ donor mice into CD45.2$^+$ glioma-bearing animals. After tumor explantation and isolation of tumor-infiltrating immune cells, we detected a CD45.1$^+$ chNKG2D T cell population by flow cytometry (Figure 3B and Suppl. Figure 3). Additionally, the infiltration of intracranial tumors with CD45.1$^+$ chNKG2D T cells was verified by immunohistochemistry (Figure 3C). Compared to direct intratumoral injection which served as a control, we detected fewer CD45.1$^+$ cells after intravenous injection, indicating that only a fraction of the administered T cells migrates to and stays at the tumor site (Figure 3B, C).

NKG2D-based CAR T cells prolong survival of syngeneic orthotopic glioma-bearing mice

To explore the efficacy of NKG2D-based CAR T cell therapy against experimental gliomas *in vivo*, we intravenously injected chNKG2D or wtNKG2D T cells on days 5, 7 and 10 after orthotopic tumor implantation. This resulted in a significantly prolonged survival of GL-261 glioma-bearing mice and cured 22% of the animals as confirmed by MRI and long-term follow-up (Figure 4A). Systemic administration of CAR T cells may be associated with on-target off-tumor activity and thus local or systemic toxicity. We did not observe weight loss as an indirect indicator for toxicity following repetitive systemic administration of chNKG2D or wtNKG2D T cells (Figure 4B). According to the mouse gene expression database and the BioGPS gene expression database, NKG2D ligands are generally expressed at low levels in normal tissues with the highest expression of RAE-1 in liver, hematopoietic system and reproductive system (Suppl. Figure 4A and B). We observed no changes in liver enzyme amounts in sera of mice treated repetitively with chNKG2D or wtNKG2D T cells. Furthermore, we did not observe differences in peripheral blood counts (Figure 4B).

To investigate the potential treatment effect and tolerability in the setting of an unrestricted migration to the tumor site, we injected chNKG2D T cells directly intratumorally in glioma-bearing mice. This application route significantly prolonged the survival after a single injection of chNKG2D T cells in a dose-dependent manner and increased the fraction of cured mice compared to intravenous CAR T cell administration (Figure 4C).

Glioma-bearing mice surviving after chNKG2D T cell treatment are long-term protected against tumor re-challenge

An impressive feature of cancer immunotherapy is the potential for a long-lasting treatment effect (32). We therefore determined a potential long-term immune protection in glioma-bearing mice that had survived following chNKG2D CAR T cell treatment. To this end, GL-261 glioma cells were implanted into the contralateral hemisphere 5-8 months after the first implantation. No additional treatment was administered. All of these mice that survived after the initial CAR T cell treatment also survived the tumor re-challenge, and at day 18 after tumor implantation, no tumor mass was detectable by MRI (Figure 5A and B). In contrast, all naive mice had large tumors and finally had to be euthanized. In order to investigate the mechanism underlying this long-term protection, we re-challenged the long-term survivors a second time and isolated lymphocytes from the brain and from cervical, axillary and inguinal lymph nodes three days after this second tumor re-challenge. A prominent $CD8^+$ T cell population was detected within the re-challenged hemisphere of chNKG2D T cell-treated long-term survivors but not in naive mice (Figure 5C). The brain-resident $CD8^+$ T cell population predominantly secreted IFN-γ in response to GL-261 glioma cells, as demonstrated by FACS-sorting of brain-resident $CD4^+$ and $CD8^+$ T cells from long-term survivors or naïve tumor-bearing mice three days after tumor re-challenge and *ex vivo* stimulation with GL-261 cells or MHC-matched but non-glioma EL-4 cells (Suppl. Figure 5). There was no difference in intermediate and high CD44-expressing $CD4^+$ and $CD8^+$ T cells between lymph nodes of re-challenged chNKG2D T cell-treated long-term survivors and those of naive tumor-bearing mice (Figure 5D). This suggests a local long-term protection independent from a peripheral memory response. In line with these findings, we noticed an equivalent tumor cell cytolysis and IFN-γ production after co-culture of GL-261 cells with T cells isolated

from lymph nodes of re-challenged chNKG2D T cell-treated long-term survivors or T cells from naive tumor-bearing mice. We detected *chNKG2D* transcripts in brains from long-term survivors but not from naive mice and not in peripheral lymph nodes or spleens from long-term survivors or naïve mice (Figure 5D), which suggests local persistence of NKG2D CAR T cells in the CNS.

Irradiation increases the therapeutic activity of NKG2D-based CAR T cells against gliomas

There is an intense discussion on the implementation of immunotherapy into conventional treatment regimens. Radiotherapy is part of the standard treatment of gliomas (33). We have previously observed an upregulation of NKG2D ligands on the glioma cell surface upon irradiation (23). This built the rationale to combine radiotherapy with NKG2D-based CAR T cell therapy. A single cranial irradiation with 4 Gy at day 7 after tumor implantation had no effect on survival when given alone but further increased the activity of NKG2D-based CAR T cells against orthotopic gliomas as demonstrated by synergistically prolonged survival in 2 independent glioma models, reduced tumor volume measured by MRI, and an increased fraction of long-term surviving mice in the SMA-560 glioma model (Figure 6A-D). We identified two underlying mechanisms for this synergistic activity. Low-dose pre-irradiation of glioma cells and subsequent co-culture with chNKG2D- or wtNKG2D-expressing T cells resulted in an increased cytolysis and IFN-γ production *in vitro* (Figure 7A). Also *in vivo*, local tumor irradiation increased the IFN-γ expression of tumor-infiltrating chNKG2D T cells (Figure 7B, Suppl. Figure 6A). This points towards a direct tumor-cell related effect of irradiation that boosts NKG2D CAR T cell activity, which can be attributed due to the induction of NKG2DL (Suppl. Figure 6B) (23). Moreover, we observed an indirect migration-related mechanism by tracking

fluorescently labeled chNKG2D CAR T cells after intravenous injection. Here, we noticed an increased accumulation of CAR T cells upon irradiation (Figure 7C) suggesting that local subtherapeutic irradiation promotes the migration of CAR T cells to the tumor site.

Discussion

CAR T cell therapy is an emerging immunotherapy that is under development against several malignancies including glioblastoma. However, the potentially immunosuppressive microenvironment and intraparenchymal location of solid tumors represent challenges which require a systematic development of strategies boosting the anti-tumor efficacy of CAR T cells against these tumors (34). Furthermore, it remains to be determined how immunotherapy with CAR T cells can be integrated at its best into conventional cancer therapies.

We addressed these questions in orthotopic, fully-immunocompetent, preclinical glioblastoma models. Except for one report (35), all preclinical studies published so far have assessed CAR T cells against experimental gliomas in a xenograft setting. However, only a syngeneic setting which involves an intact immune system and a physiological tumor microenvironment allows appropriately assessing the challenges of CAR T cell therapy against solid tumors including potential off-tumor toxicities.

In contrast to other single target antigens for CAR T cell therapy that are currently being investigated against glioblastoma, the NKG2D CAR elegantly targets multiple tumor-associated ligands. This may decrease the probability of tumor immune escape from CAR T cell treatment due to antigen loss (17).

We demonstrate strong anti-tumor activity of NKG2D-based CAR T cells against glioma cells *in vitro* in a syngeneic setting (Figures 1, 2). Furthermore, *in vivo* tracking of CAR T cells verifies homing of CAR T cells to the intracerebral tumor site after systemic CAR T cell administration (Figure 3). The target antigens of the NKG2D CAR are not exclusively expressed on tumor cells but can be induced also in non-tumoral tissues by unphysiological cell stress or inflammation (36-38). Although NKG2D ligand expression by other tissues may result in *on-target off-tumor* toxicity,

we did not observe any signs of toxicity upon systemic CAR T cell administration (Figure 4B) at the level of body weight, liver function tests or peripheral blood counts. In line with these findings, a first-in-human phase I trial assessing NKG2D-based CAR T cells against hematological malignancies has completed enrollment of a first cohort of patients without treatment-related safety issues (39).

Treatment of glioma-bearing mice with NKG2D CAR T cells resulted in a significant proportion of surviving mice which were long-term protected against tumor re-challenge in the contralateral hemisphere (Figures 5A, B). In contrast to previous studies (28,35), this may not only be the result of a classical memory T cell response, but probably it may also involve long-term persistence of NKG2D CAR T cells in the brain as demonstrated by detection of the *chNKG2D* transcripts in the brain tissue of long-term surviving mice 8 months after initial CAR T cell treatment (Figure 5D). It remains an open question why NKG2D CAR T cells seem to persist in the CNS but not in extracerebral organs. Possibly, the CNS as an immune-privileged site provides a niche for homeostatic proliferation of tissue-resident T cells which allows CAR T cell persistence.

Future glioblastoma treatment regimens may rely on combination therapies and may implement novel immunotherapeutic strategies such as CAR T cell administration. The combination of immunotherapy with conventional anti-cancer treatments such as radiotherapy may result in synergistic activity (40,41). However, the combination of CAR T cell therapy with radiotherapy has only been investigated with total body irradiation used as a myeloablative host-conditioning regimen before administration of CAR T cells (35). Our data demonstrate synergistic anti-tumor activity of local tumor irradiation and NKG2D CAR T cell therapy (Figure 6). Mechanistically, this combination produces stronger CAR T cell activity upon recognition of irradiated

tumor cells and an improved trafficking of intravenously injected CAR T cells to the tumor site (Figure 7).

In summary, this study highlights the potential of NKG2D CAR T cells as a promising therapeutic approach against glioblastoma. Long-term tumor control due to persistence of these cells at the tumor site and synergistic activity in combination with radiotherapy suggest that NKG2D CAR T cells represent a novel therapeutic option that warrants clinical evaluation in glioma patients.

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Figure legends

Figure 1. **NKG2D CAR T cells lyse glioma cells in a NKG2D-dependent manner.** The murine glioma cell lines GL-261 (A), SMA-497 (B), SMA-540 (C) or SMA-560 (D) were used as target cells in 4 h cytolysis assays. T cells from C57BL/6 mice (for GL-261) or VM/Dk mice (for SMA-497, SMA-540 and SMA-560) were transduced with chNKG2D (CH) or wtNKG2D (WT) and used as effector cells at various effector : target (E:T) ratios (corresponding upper graphs for each cell line). Blocking anti-NKG2D or isotype control antibodies were used to pre-incubate chNKG2D or wtNKG2D T cells for 1 h before using them as effector cells at an E:T of 40:1 (corresponding lower graphs for each cell line). Data are presented as mean ± SD (*$p < 0.05$; **$p < 0.01$).

Figure 2. **NKG2D-based CAR T cells produce high levels of IFN-γ upon co-culture with glioma cells.** The murine glioma cell lines GL-261 (A), SMA-497 (B), SMA-540 (C) or SMA-560 (D) were co-cultured with chNKG2D- or wtNKG2D-expressing T cells. After 6 h of co-culture, IFN-γ levels were determined in $CD4^+$ and $CD8^+$ T cells by flow cytometry. Bar plots show mean and SD from 2 independent experiments (*$p < 0.05$; **$p < 0.01$). FACS plots show data from one representative experiment and numbers indicate percentage of IFN-γ-positive T cells.

Figure 3. **NKG2D-based CAR T cells migrate to intracranially growing gliomas after systemic administration.** A. ChNKG2D T cells were labeled with CellBrite NIR790. Subsequently, 10^6 labeled cells (+ctrl) or unlabeled cells (PBS, - ctrl respectively) were injected intracranially in the brain of a non-tumor-bearing mouse and the signal from labeled T cells was detected at the tumor site by fluorescence

molecular tomography (FMT) (left). Tumor-bearing mice were treated with a single i.v. injection of 5 x 10^6 chNKG2D T cells at day 5 after tumor cell implantation. The near-infrared signal was acquired at the tumor site by FMT at the indicated time points after injection (right). Color scales indicate signal intensities. B. Five x 10^6 CD45.1$^+$ chNKG2D T cells were injected either intravenously or intratumorally at a single time point in CD45.2$^+$ tumor-bearing mice at day 5 after tumor implantation. Three days later, tumor-infiltrating immune cells were isolated from the tumor-bearing hemisphere and CD45.1$^+$ and CD45.2$^+$ cells were detected by flow cytometry. C. The animals were treated as in (B) and CD45.1$^+$ cells were detected by immunohistochemistry after removal of the brains at day 10. CD45.1$^+$ T cells are stained in brown. Spleen sections from CD45.1$^+$ mice were used as positive control. One representative image out of 3 different mice per group is shown.

Figure 4. **NKG2D-based CAR T cell treatment confers a survival benefit in syngeneic orthotopic glioma-bearing mice.** A. GL-261 tumor-bearing mice were treated intravenously with 5 x 10^6 chNKG2D- or wtNKG2D-expressing T cells on days 5, 7 and 10 after tumor implantation. Survival data are presented as Kaplan-Meier plots (left). Representative T2w MRI images of mice receiving wtNKG2D- (upper panel) or chNKG2D T cells (lower panel) at day 15 after tumor implantation are shown (right). The white arrow indicates the tumor. B. Body weight was determined every other day (upper left), liver enzymes (upper right) were assessed at day 16 and peripheral blood counts were assessed at day 14 of GL-261 tumor-bearing mice treated intravenously with 5 x 10^6 chNKG2D or wtNKG2D T cells at days 5, 7 and 10 after tumor-implantation. C. GL-261 tumor-bearing mice were treated with a single intratumoral injection of 2 x 10^4, 2 x 10^5 or 2 x 10^6 chNKG2D T cells or PBS control. Survival data are presented as Kaplan-Meier plots (left). P

values were calculated with log-rank test (*p < 0.05; **p < 0.01). Representative T2w MRI images of mice receiving wtNKG2D- (upper panel) or $2x10^6$ chNKG2D T cells (lower panel) at day 15 after tumor implantation are shown (right). The white arrow indicates the tumor.

Figure 5. **Glioma-bearing mice surviving after NKG2D CAR T cell treatment are long-term protected against tumor re-challenge.** Long-term surviving mice cured by systemic (i.v.) or intratumoral (i.t.) administration of chNKG2D T cells were re-challenged 6 months after the initial tumor implantation with GL-261 cells in the contralateral hemisphere. As a control, GL-261 cells were inoculated into naive mice. A. Kaplan Meier survival curves for the 3 cohorts are indicated. B. T2w MRI scans at day 18 after tumor implantation are shown. The white arrow indicates the tumor. The upper panel represents images from naive control mice and the lower panel represents images from re-challenged chNKG2D T cell long-term survivors following tumor implantation. C. Long-term surviving mice received a second tumor re-challenge (2 months after the first re-challenge) and three days after tumor (re)-implantation, tumor-infiltrating $CD4^+$ and $CD8^+$ T cells were isolated and analyzed by flow cytometry. An individual plot of tumor-infiltrating lymphocytes from one mouse is shown on the left and a diagram depicting the mean and SD of 3 mice shown on the right (*p < 0.05; **p < 0.01). D. Same setup as in (C) and isolation of cervical, axillary and inguinal lymph nodes at day 3 after the second tumor re-challenge. Effector (Teff) and memory (Tmem) T cells were separated using flow cytometry and CD44 expression levels. Populations with intermediate (effector T cells) or high (memory T cells) CD44 expression in the $CD4^+$ or $CD8^+$ T cell compartments are indicated (left). The isolated lymph node-derived T cells were used as effector cells in a 4 h immune cell lysis assay or IFN-γ ELISPOT with fresh GL-261 cells as target cells (right upper

panel). Real-time PCR for *chNKG2D* was performed after RNA isolation and cDNA preparation from brains, spleens and peripheral lymph nodes isolated 3 days after tumor-challenge of naive control mice or 3 days after second re-challenge of long-term surviving mice 8 months after initial NKG2D CAR T cell treatment (right lower panel, with n.d. corresponding to non-detectable).

Figure 6. **Irradiation and systemic NKG2D-based CAR T cell treatment act synergistically against experimental gliomas.** GL-261 (A and C) or SMA-560 (B and D) tumor-bearing mice were treated intravenously with 5 x 10^6 chNKG2D (CH) or wtNKG2D (WT) T cells on days 5, 7 and 10 after tumor-implantation either alone or with a single local irradiation with 4 Gy at day 7 after tumor implantation. A, B. Kaplan Meier curves are shown and p values were calculated with log-rank test (**p < 0.01 for WT vs. CH; ***p < 0.001 for WT+IR vs. CH+IR). C. Representative MRI scans at day 15 of ~~5~~ 3 animals of each of the 4 cohorts of GL-261 tumor-bearing mice are shown (left). The white arrow indicates the tumor. Tumor volumes (right) were calculated based on MRI analyses using the formula H x W x L/2 (*p < 0.05 and **p < 0.01 for WT vs. CH or WT+IR vs. CH+ IR, $^{+}$p < 0.05 for CH vs. CH+IR). D. Same setting as in C but for SMA-560 tumor-bearing mice.

Figure 7. **Irradiation-mediated boosting of CAR T cell activity relies on direct effects on tumor cells as well as increased CAR T cell migration.** A. GL-261 cells, pre-irradiated (+IR) 24 h prior to the assay with 4 Gy or not were used as target cells in cytotoxicity assays using chNKG2D (CH) or wtNKG2D (WT) T cells as effector cells at the indicated E:T ratios (left). After 4 h of co-culture at a ratio of 40:1, IFN-γ levels were determined by intracellular cytokine staining and flow cytometric assessment (right). Mean and SD are shown (*p < 0.05; **p < 0.01 for WT vs. CH

and $^{+}p < 0.05$; $^{++}p < 0.01$ for WT/CH vs. WT/CH + IR). B. 5 x 10^6 $CD45.1^+$ wtNKG2D or chNKG2D were i.v. injected on days 5, 7, 10 after implantation of GL-261 tumors in $CD45.2^+$ mice. In addition, mice were irradiated (IR) at day 7 with a single local dose of 4 Gy or not. At day 12 after tumor implantation, brain-infiltrating immune cells were isolated and IFN-γ expression assessed in $CD45.1^+$ cells by flow cytometry. Mean and SD from 3 mice is shown with $^{**}p < 0.01$ for WT vs. CH and WT + IR vs. CH+IR and $^{+}p < 0.05$; for WT/CH vs. WT/CH + IR. C. ChNKG2D T cells were labeled with CellBrite NIR790. Subsequently, 5 x 10^6 labeled cells were i.v. injected on days 5, 7 and 10 after tumor implantation. In addition, mice were irradiated (IR) at day 7 with a single local dose of 4 Gy or not. The near-infrared signal was acquired at the tumor-site by FMT at the following time points: T1 = 24 h prior to IR, T2 = 24 h post IR, T3 = 144 h post IR. Representative images are shown on the left and a quantification of the detected signal on the right ($^{*}p < 0.05$; $^{**}p < 0.01$)

Fig. 1

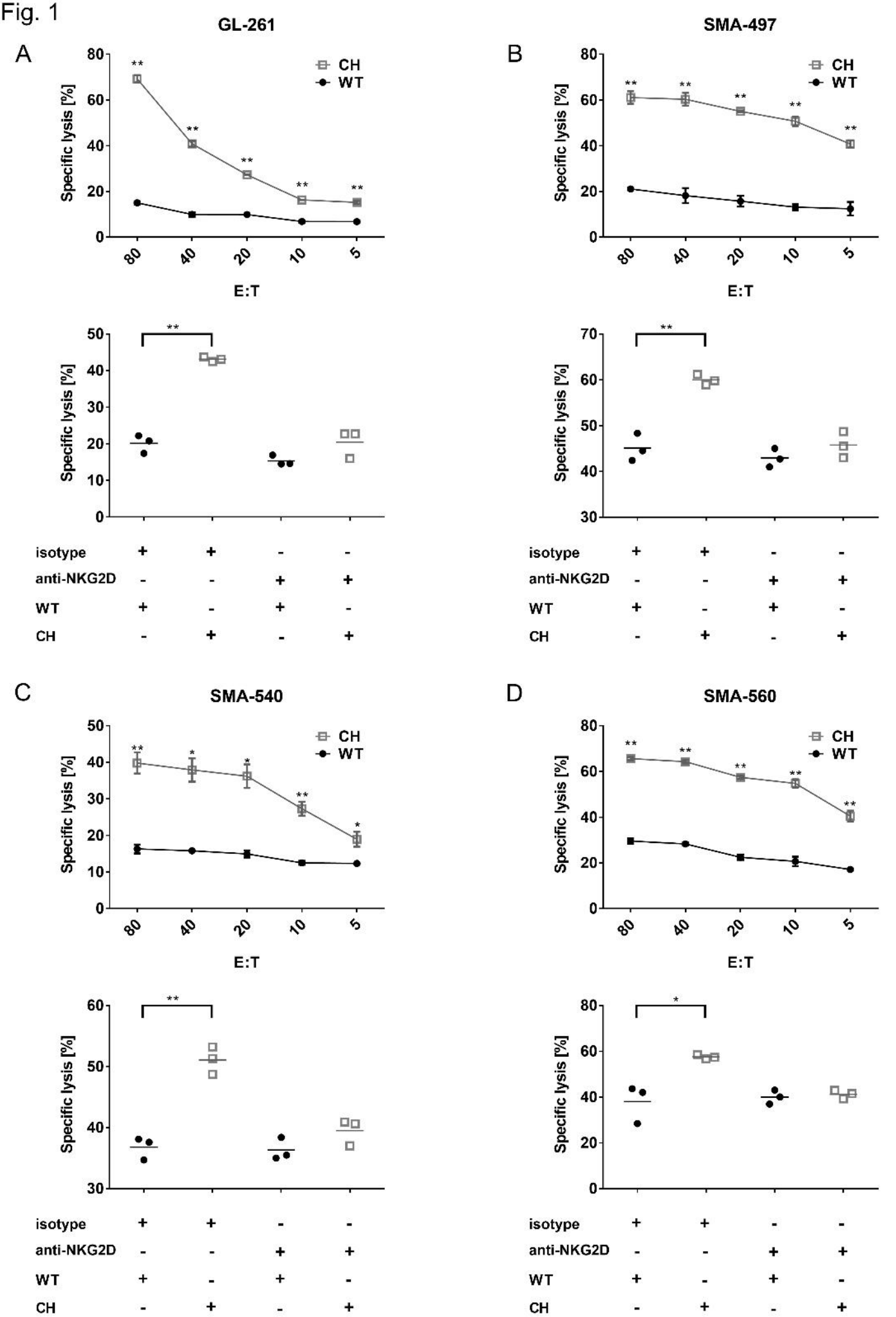

A
GL-261
Specific lysis [%]
CH
WT
E:T
isotype
anti-NKG2D
WT
CH
B
SMA-497
C
SMA-540
D
SMA-560

Fig. 2

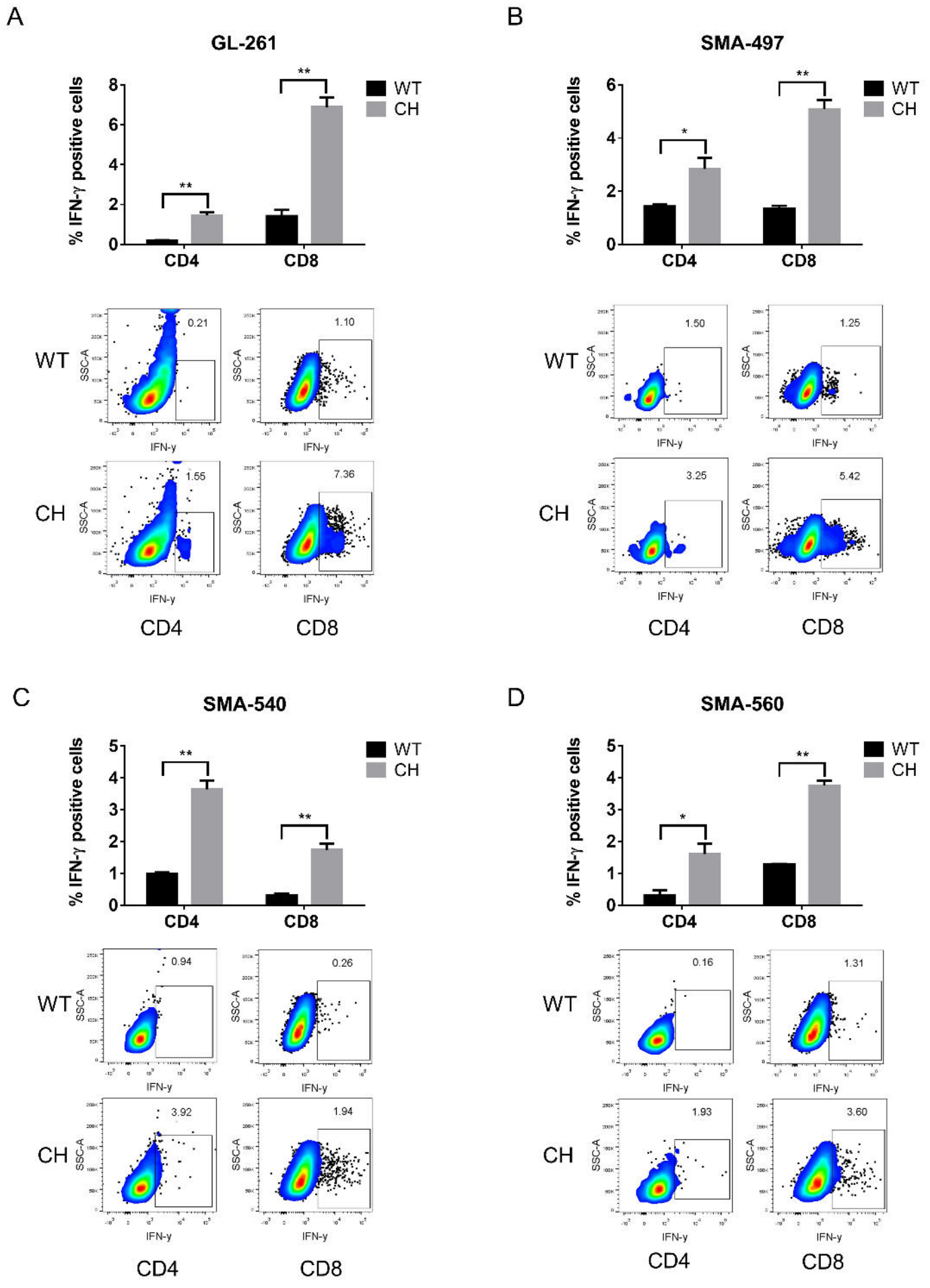
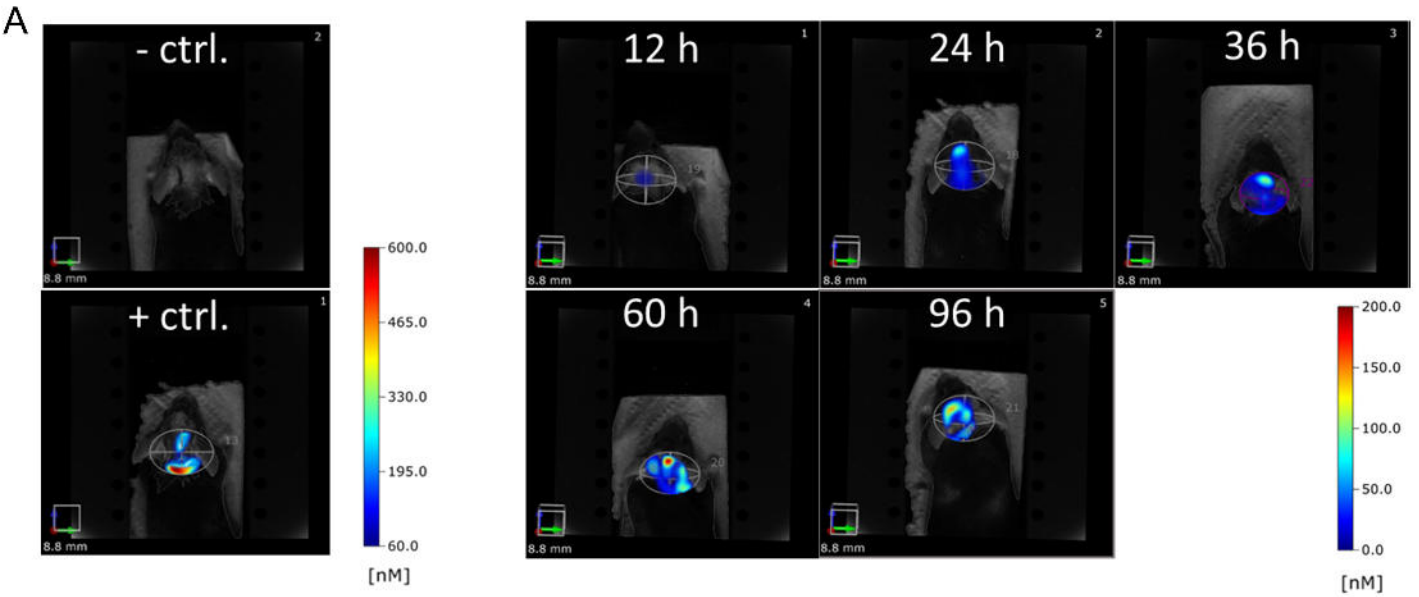
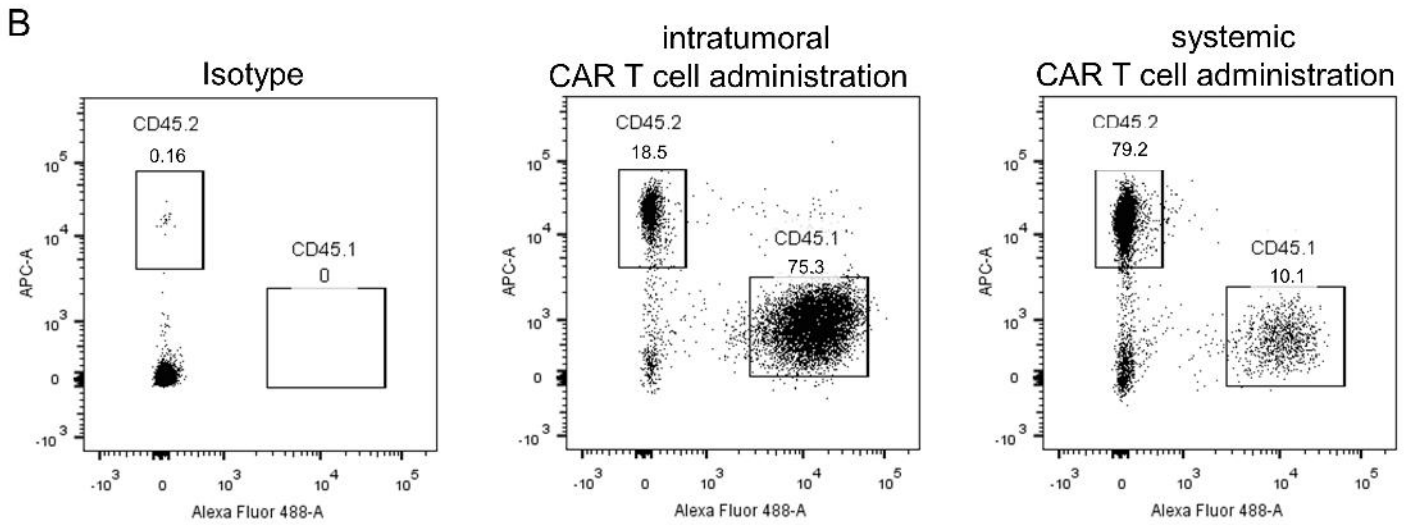

A
GL-261
% IFN-γ positive cells
WT
CH
CD4
CD8
0.21
1.10
1.55
7.36
SSC-A
IFN-γ
B
SMA-497
1.50
1.25
3.25
5.42
C
SMA-540
0.94
0.26
3.92
1.94
D
SMA-560
0.16
1.31
1.93
3.60

Fig. 3


A
- ctrl.
+ ctrl.
600.0
465.0
330.0
195.0
60.0
[nM]
12 h
24 h
36 h
60 h
96 h
200.0
150.0
100.0
50.0
0.0
[nM]


B
Isotype
CD45.2
0.16
CD45.1
0
APC-A
Alexa Fluor 488-A
intratumoral
CAR T cell administration
CD45.2
18.5
CD45.1
75.3
systemic
CAR T cell administration
CD45.2
79.2
CD45.1
10.1

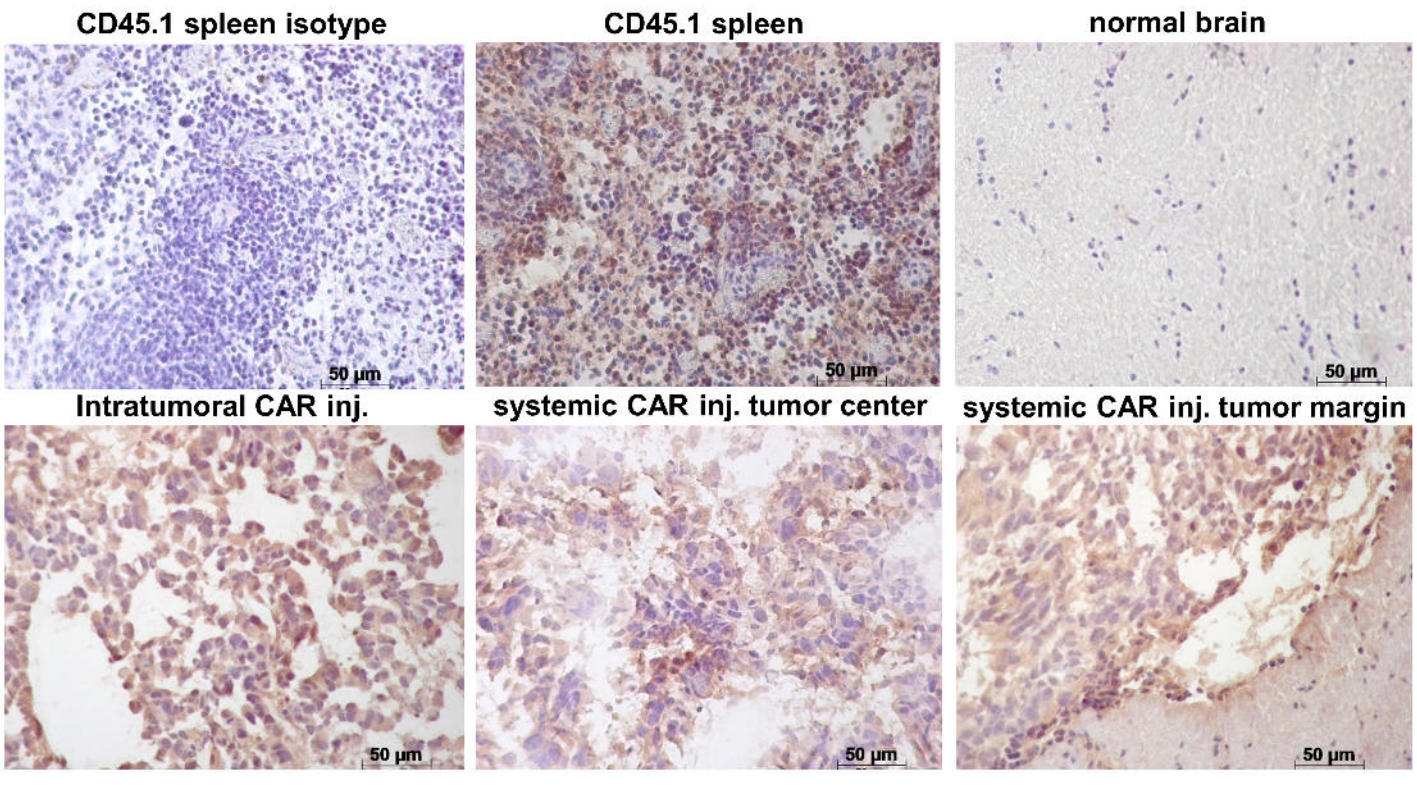

C
CD45.1 spleen isotype
CD45.1 spleen
normal brain
Intratumoral CAR inj.
systemic CAR inj. tumor center
systemic CAR inj. tumor margin
50 µm

Fig. 4

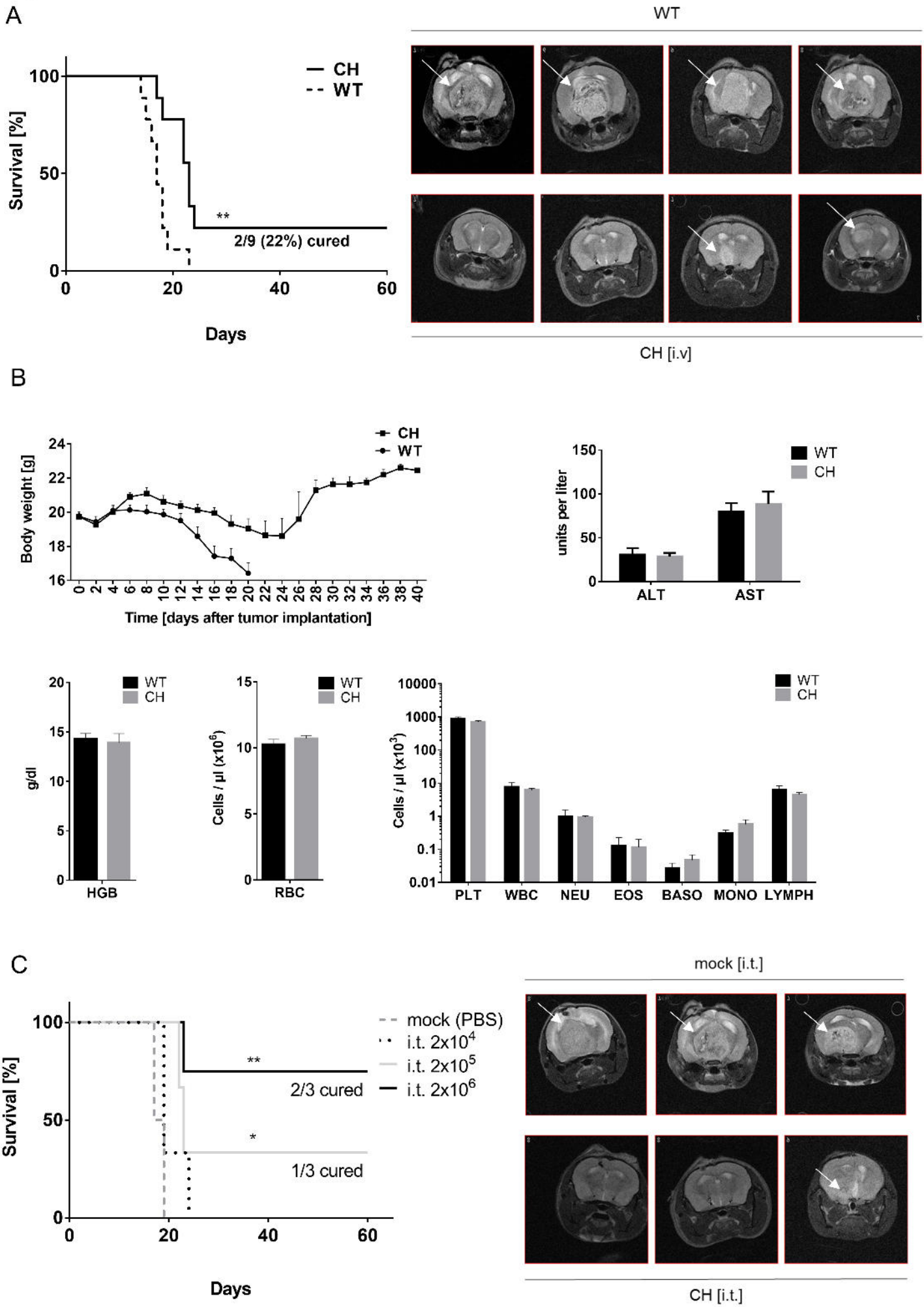
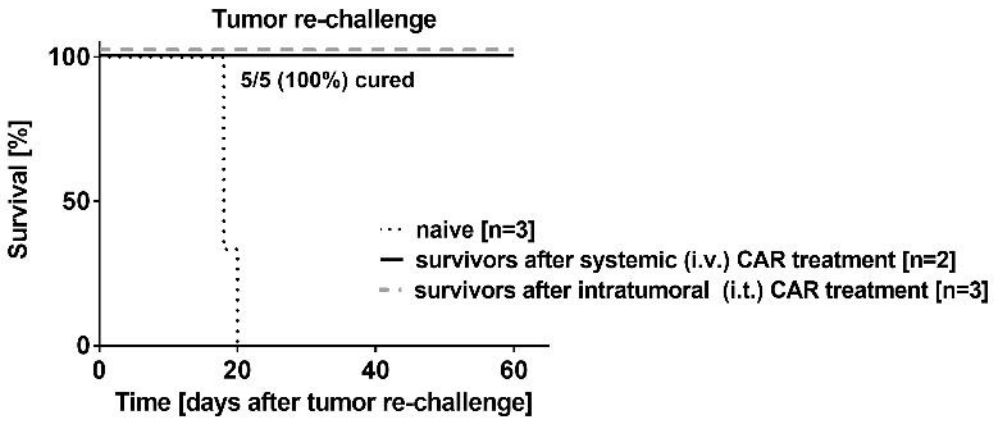
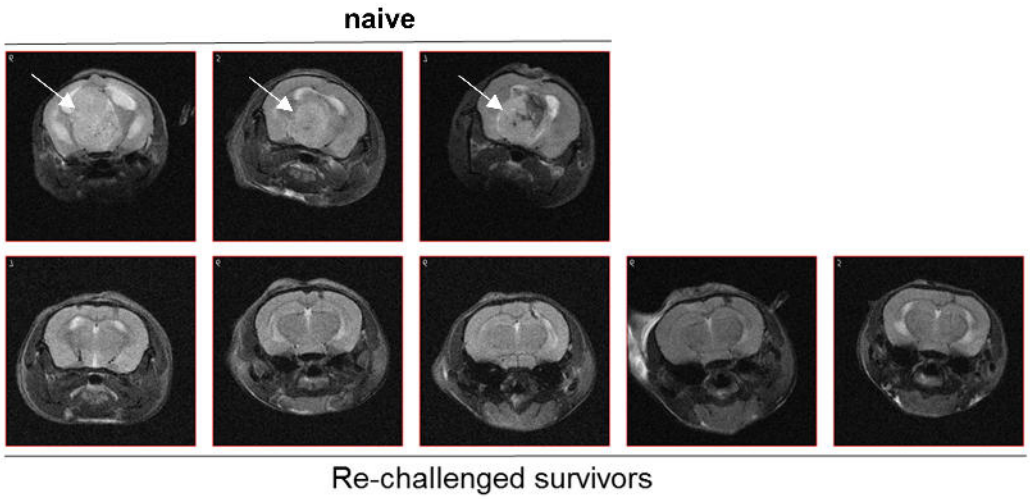

A
WT
CH
WT
Survival [%]
100
50
0
0
20
40
60
Days
**
2/9 (22%) cured
CH [i.v]
B
CH
WT
Body weight [g]
24
22
20
18
16
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Time [days after tumor implantation]
WT
CH
units per liter
150
100
50
0
ALT
AST
WT
CH
g/dl
20
15
10
5
0
HGB
WT
CH
Cells / µl (x10^6)
15
10
5
0
RBC
WT
CH
Cells / µl (x10^3)
10000
1000
100
10
1
0.1
0.01
PLT
WBC
NEU
EOS
BASO
MONO
LYMPH
C
mock [i.t.]
mock (PBS)
i.t. 2x10^4
i.t. 2x10^5
i.t. 2x10^6
Survival [%]
100
50
0
0
20
40
60
Days
**
2/3 cured
*
1/3 cured
CH [i.t.]

Fig. 5

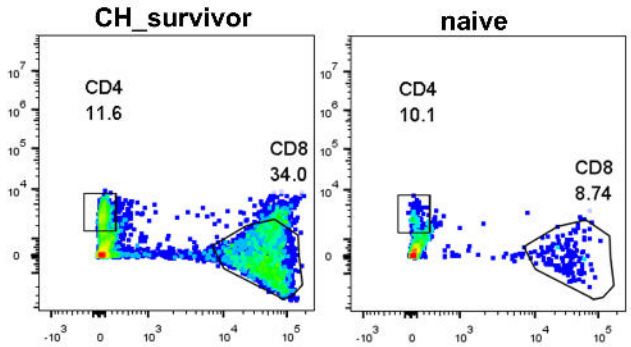
A

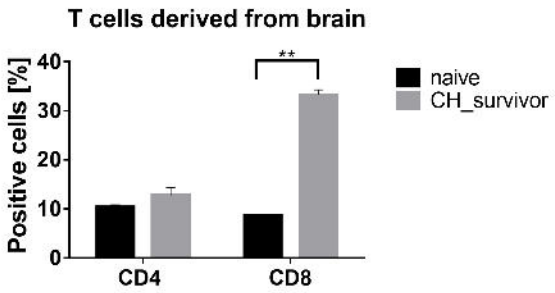

Tumor re-challenge
100
50
0
Survival [%]
5/5 (100%) cured
naive [n=3]
survivors after systemic (i.v.) CAR treatment [n=2]
survivors after intratumoral (i.t.) CAR treatment [n=3]
0
20
40
60
Time [days after tumor re-challenge]

B

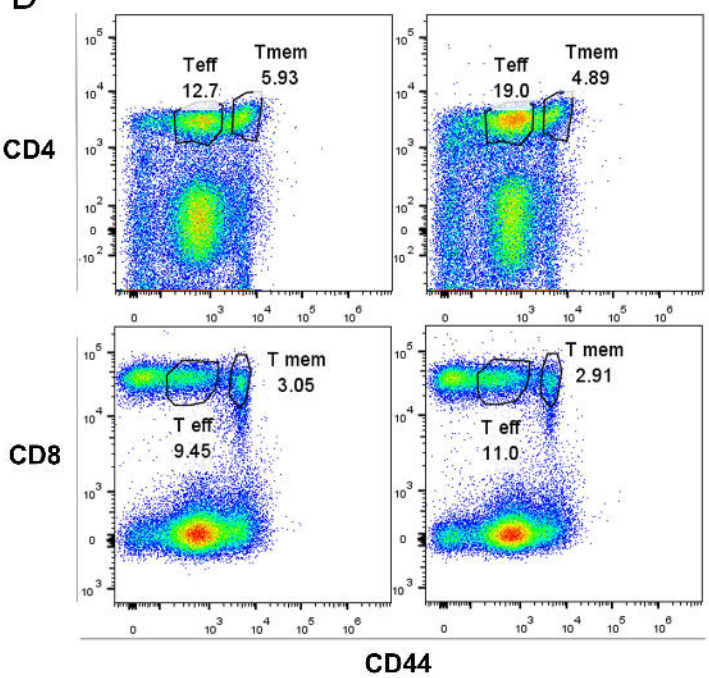

naive
Re-challenged survivors

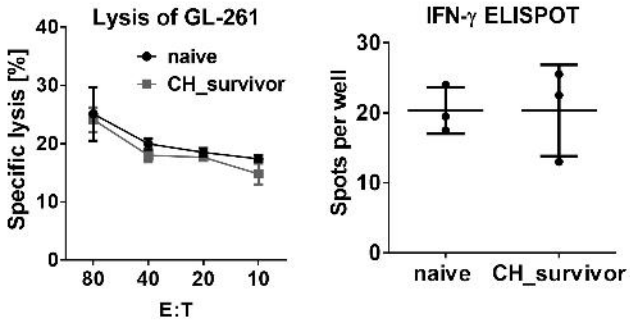
C


CH_survivor
naive
CD4
11.6
CD8
34.0
CD4
10.1
CD8
8.74


T cells derived from brain
Positive cells [%]
40
30
20
10
0
**
naive
CH_survivor
CD4
CD8

D


CD4
Teff
12.7
Tmem
5.93
Teff
19.0
Tmem
4.89
CD8
T mem
3.05
T eff
9.45
T mem
2.91
T eff
11.0
CD44


Lysis of GL-261
Specific lysis [%]
40
30
20
10
naive
CH_survivor
80
40
20
10
E:T
IFN-γ ELISPOT
Spots per well
30
20
10
0
naive
CH_survivor

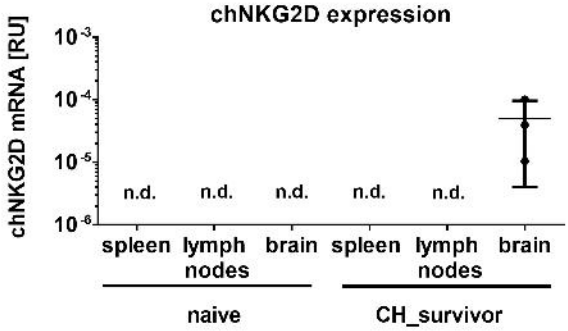
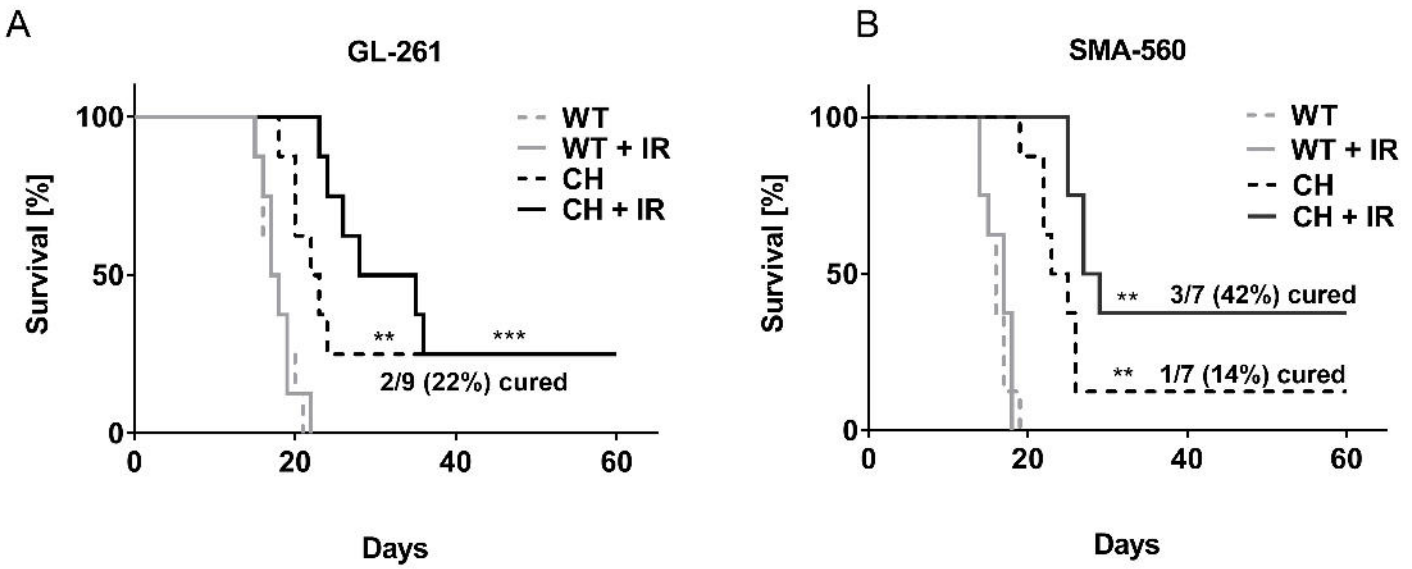

chNKG2D expression
chNKG2D mRNA [RU]
10-3
10-4
10-5
10-6
n.d.
n.d.
n.d.
n.d.
n.d.
spleen
lymph nodes
brain
spleen
lymph nodes
brain
naive
CH_survivor

Fig. 6


A
GL-261
Survival [%]
100
50
0
0
20
40
60
Days
WT
WT + IR
CH
CH + IR
**
***
2/9 (22%) cured
B
SMA-560
Survival [%]
100
50
0
0
20
40
60
Days
WT
WT + IR
CH
CH + IR
** 3/7 (42%) cured
** 1/7 (14%) cured

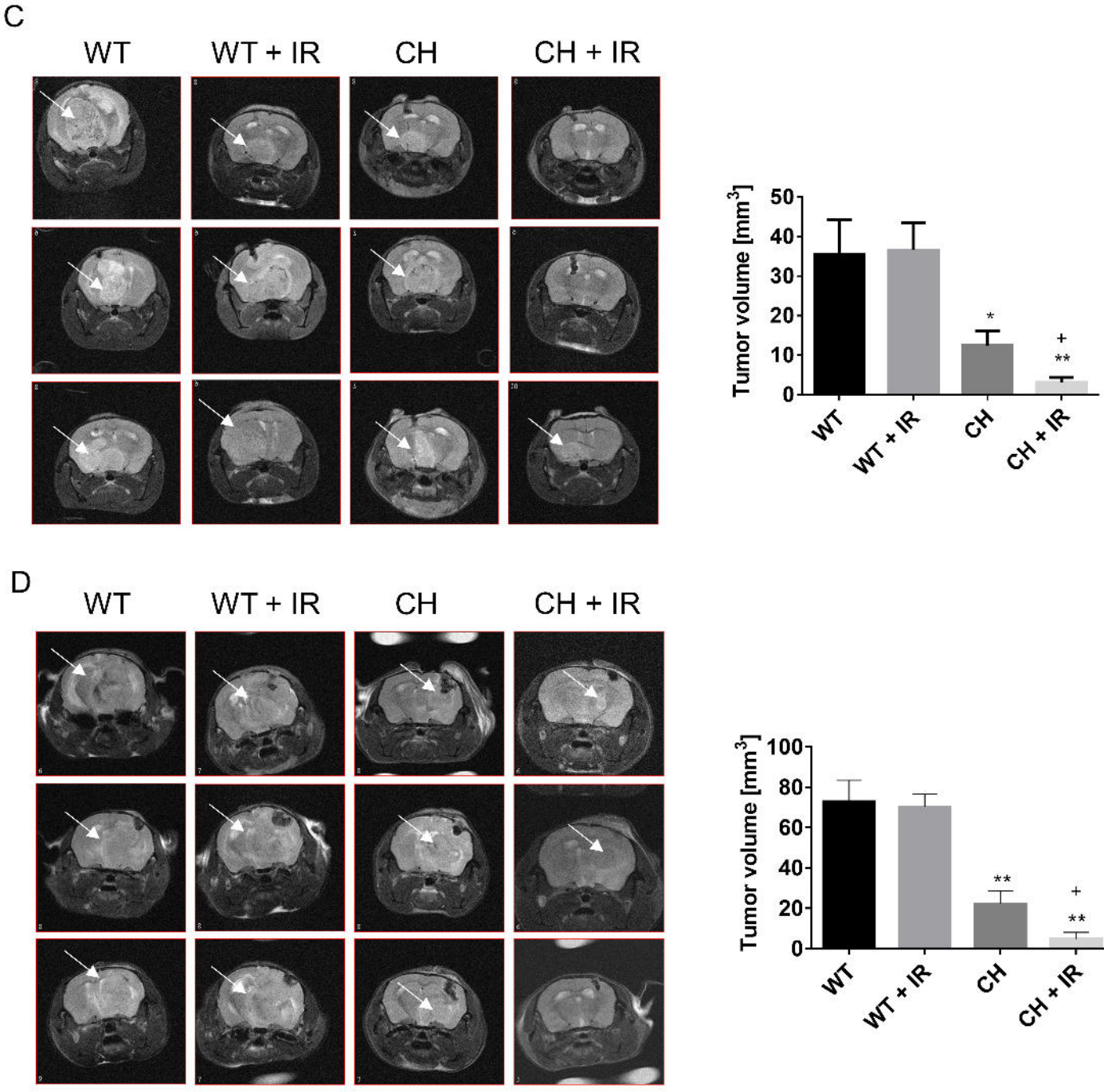
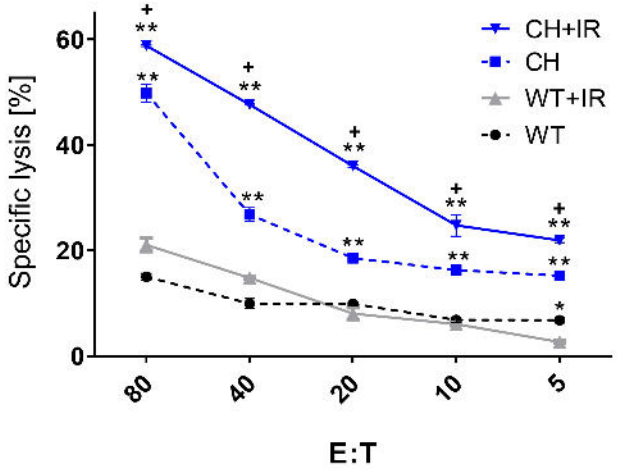
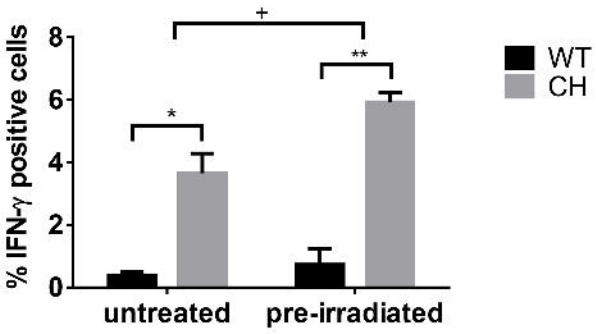

C
WT
WT + IR
CH
CH + IR
Tumor volume [mm3]
50
40
30
20
10
0
*
+
**
WT
WT + IR
CH
CH + IR
D
WT
WT + IR
CH
CH + IR
Tumor volume [mm3]
100
80
60
40
20
0
**
+
**
WT
WT + IR
CH
CH + IR

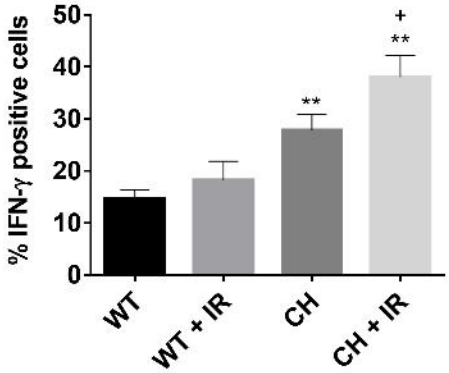
Fig. 7

A

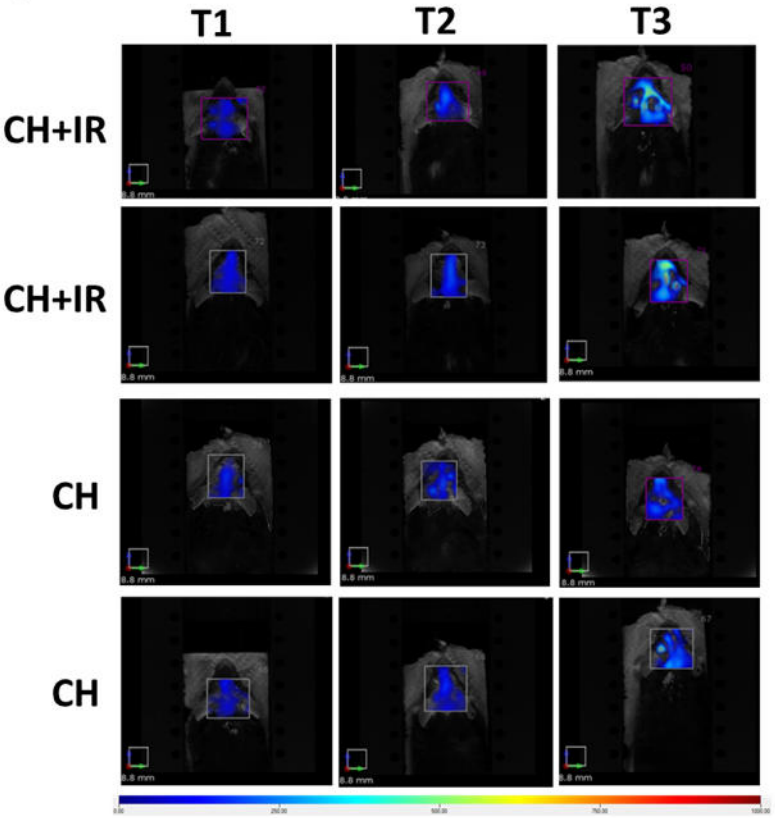

Specific lysis [%]
60
40
20
0
80
40
20
10
5
E:T
CH+IR
CH
WT+IR
WT

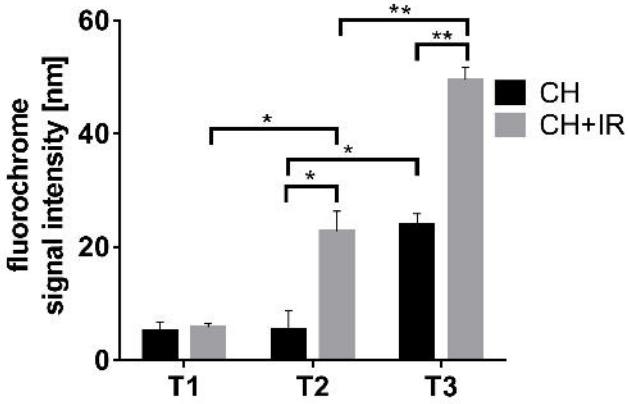

% IFN-γ positive cells
8
6
4
2
0
untreated
pre-irradiated
WT
CH

B


% IFN-γ positive cells
50
40
30
20
10
0
WT
WT + IR
CH
CH + IR

C


T1
T2
T3
CH+IR
CH+IR
CH
CH


fluorochrome signal intensity [nm]
60
40
20
0
T1
T2
T3
CH
CH+IR

Supplements

Fig.1

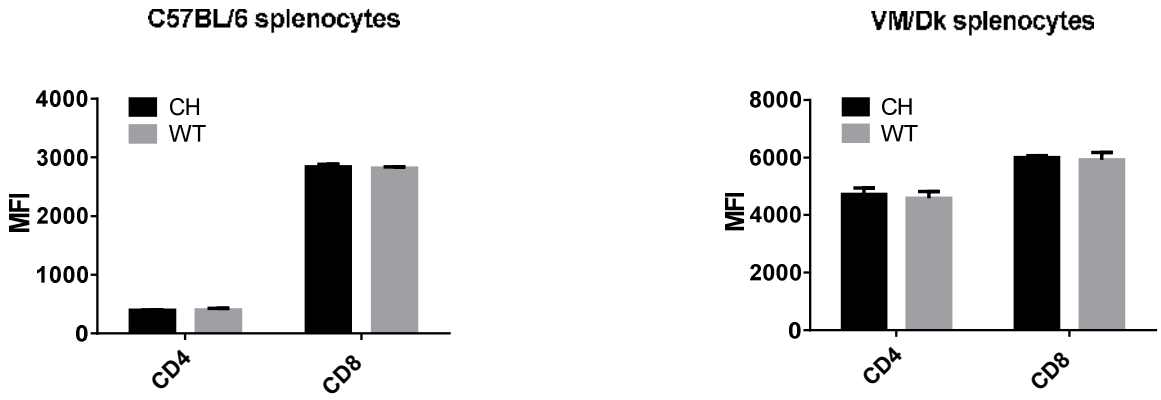


Fig. 2

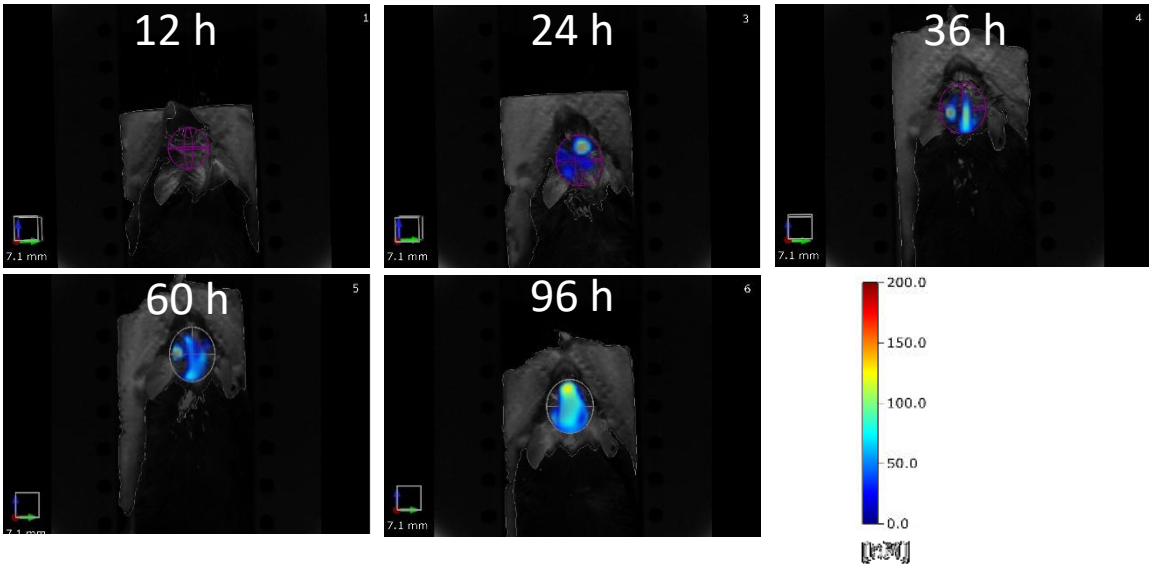


Fig. 3

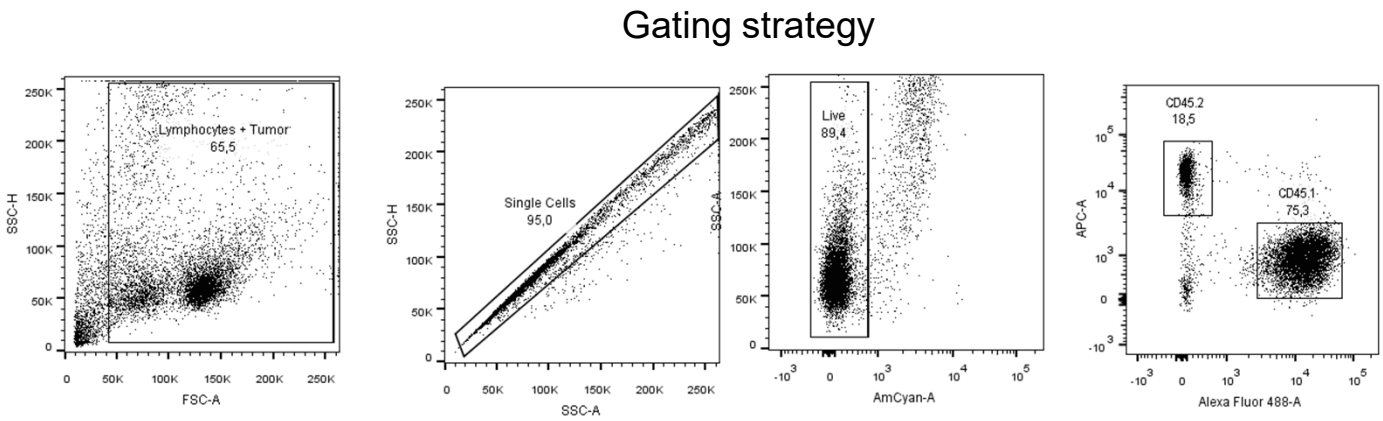
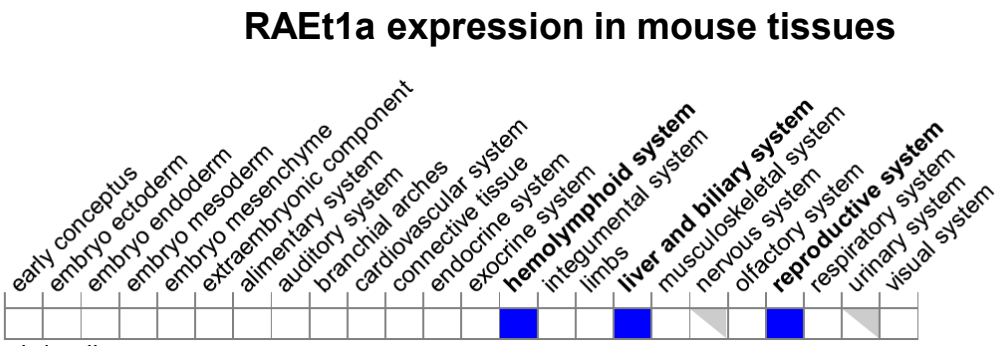
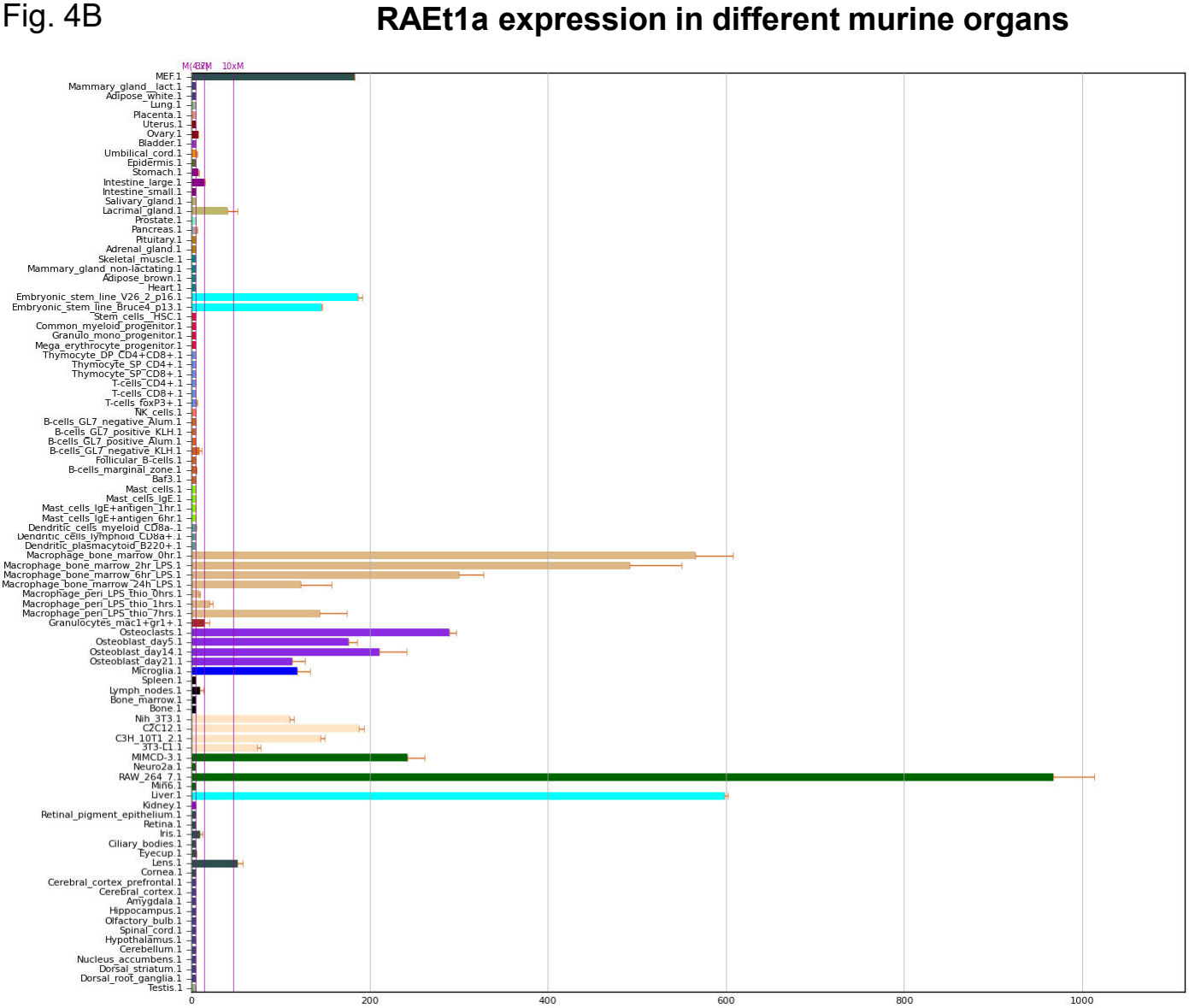


Fig. 4A



MGI- mouse gene expression database accessed 22nd october 2016

Fig. 4B



BioGPS accessed 22nd october 2016

Fig. 5

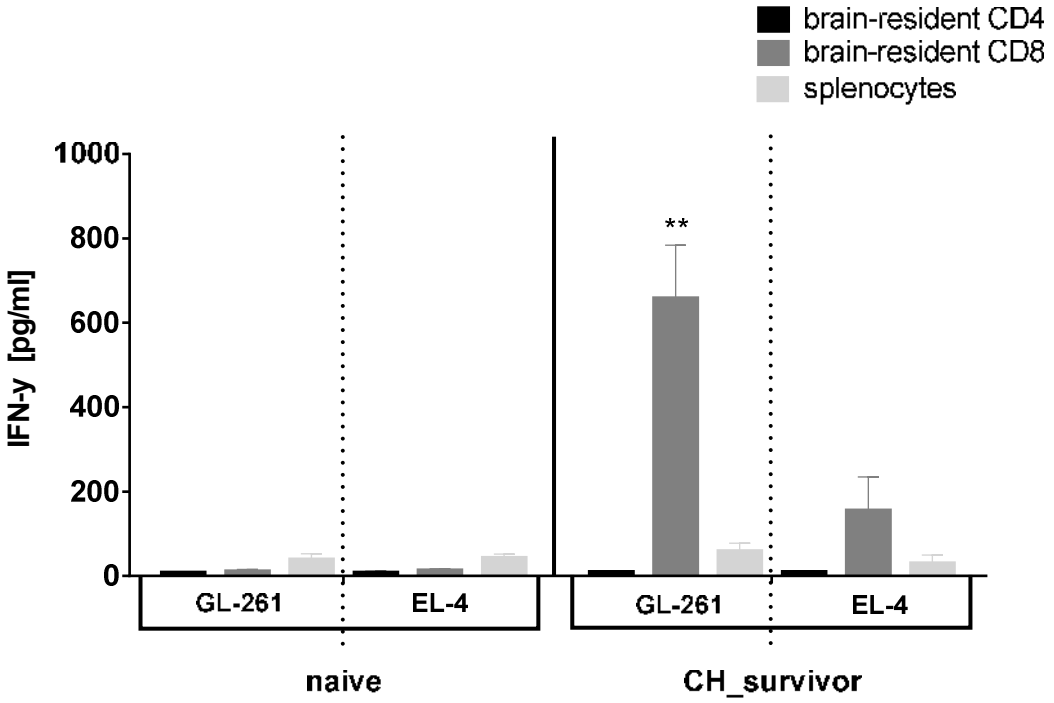

brain-resident CD4
brain-resident CD8
splenocytes
IFN-y [pg/ml]
1000
800
600
400
200
0
**
GL-261
EL-4
GL-261
EL-4
naive
CH_survivor

Fig. 6A

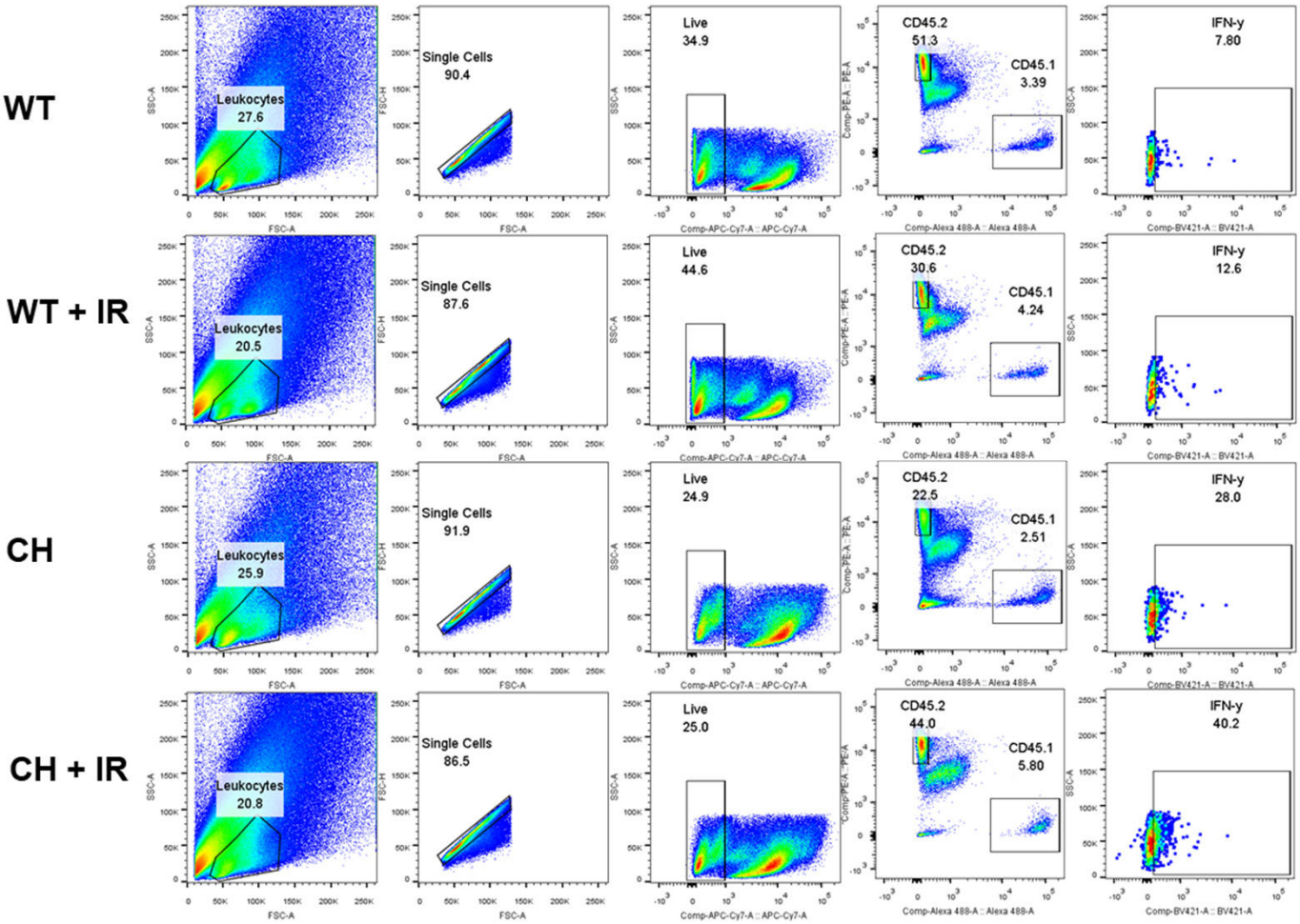
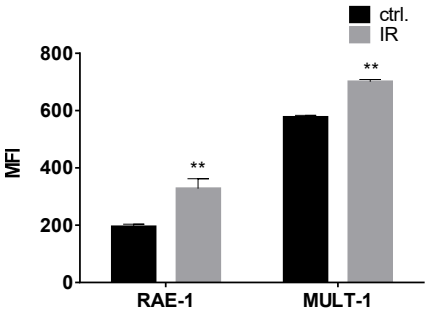

WT
WT + IR
CH
CH + IR
Leukocytes
27.6
Leukocytes
20.5
Leukocytes
25.9
Leukocytes
20.8
Single Cells
90.4
Single Cells
87.6
Single Cells
91.9
Single Cells
86.5
Live
34.9
Live
44.6
Live
24.9
Live
25.0
CD45.2
51.3
CD45.1
3.39
CD45.2
30.6
CD45.1
4.24
CD45.2
22.5
CD45.1
2.51
CD45.2
44.0
CD45.1
5.80
IFN-y
7.80
IFN-y
12.6
IFN-y
28.0
IFN-y
40.2

Fig. 6B



Supplementary Figure legends

Suppl. Fig. 1. **NKG2D cell surface expression on transduced T cells**. T cells from C57BL/6 (left) or VM/Dk (right) mice were transduced to express chNKG2D or wtNKG2D and the cell surface expression of NKG2D was assessed for $CD4^+$ and $CD8^+$ T cells by flow cytometry. Mean and SD of mean fluorescence intensity (MFI) are shown from 2 independent experiments.

Suppl. Fig. 2. **wtNKG2D T cells migrate to intracranially growing gliomas after systemic administration.** wtNKG2D T cells were labeled with CellBrite NIR790. GL-261 tumor-bearing mice were treated with a single i.v. injection of 5×10^6 wtNKG2D T cells at day 5 after tumor cell implantation. The near-infrared signal was acquired at the tumor site by FMT at the indicated time points after injection. The color scale indicates the signal intensities.

Suppl. Fig. 3. **Gating strategy of tumor-infiltrating lymphocytes.** Five $\times 10^6$ $CD45.1^+$ chNKG2D T cells were injected intratumorally at a single time point in $CD45.2^+$ tumor-bearing mice at day 5 after tumor implantation. Three days later, tumor-infiltrating immune cells were isolated from the tumor-bearing hemisphere. Detailed gating strategy of tumor-infiltrating lymphocytes comprises detection of single living, $CD45.1^+$ or $CD45.2^+$ cells.

Suppl. Fig. 4. **NKG2DL mRNA expression in normal mouse tissues.** A. The mouse gene expression database (http://www.informatics.jax.org/expression.shtml) was assessed for the expression of the main NKG2DL RAEt1a in normal mouse tissues. Blue color indicates clearly detected expression by RT-PCR and Northern blot, grey color indicates ambiguous expression. B. The BioGPS database

(http://biogps.org) (B) was assessed for the expression of the RAEt1a in normal mouse tissues. Different organ systems are color coded and displayed on the y-axis, expression levels are indicated on the x-axis.

Suppl. Fig. 5. **Brain-resident CD8 T cells from long-term surviving mice predominantly recognize GL-261 cells**. Long-term surviving mice (CH_survivor) or naïve control (naïve) mice were (re-)challenged with GL-261 cells. Three days later, splenocytes were isolated and brain-infiltrating $CD4^+$ or $CD8^+$ T cells were FACS-sorted. Five x 10^3 $CD4^+$ (black) or $CD8^+$ (dark grey) T cells or 5 x 10^4 splenocytes (grey) from long-term surviving or naïve control mice were co-cultured with 2.5 x 10^3 (in case of brain-infiltrating T cells) or 2.5 x 10^4 (in case of splenocytes) GL-261 or EL-4 cells (MHC-matched non-glioma cells). After 72 h, IFN-γ production within cell-free-media was assessed by ELISA). P value was calculated with 2-way ANOVA (**$p < 0.01$).

Supp. Fig. 6. **Local irradiation increases the effector function of chNKG2D T cells *in vivo* and induces NKG2DL**. A. Gating strategy to detect IFN-γ expression in $CD45.1^+$ chNKG2D or wtNKG2D T cells. T cells were injected intravenously at days 5, 7 and 10 after implantation of GL-261 tumor cells with or without local irradiation of 4 Gy at day 7, and brain-infiltrating immune cells were isolated at day 12 after tumor implantation. B. GL-261 tumor-bearing mice received local irradiation with 4 Gy at day 7 after tumor implantation. At day 12, cells from the tumor-bearing hemisphere were isolated and NKG2DL expression was assessed by flow cytometry. MFI and SD from 3 mice per group is shown (**$p < 0.01$).